

ANALYSIS OF TRANSCRIPTS FROM INTRACELLULAR STAGES OF *EIMERIA ACERVULINA* USING EXPRESSED SEQUENCE TAGS

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ABSTRACT: Coccidiosis in chickens is caused by 7 species of *Eimeria*. Even though coccidiosis is a complex disease that can be caused by any combination of these species, most of the molecular research concerning chicken coccidiosis has been limited to *Eimeria tenella*. The present study describes the first large-scale analysis of expressed sequence tags (ESTs) generated primarily from second-stage merozoites (and schizonts) of *E. acervulina*. In total, 1,847 ESTs were sequenced; these represent 1,026 unique sequences. Approximately half of the ESTs encode proteins of unknown function, or hypothetical proteins. Twenty-nine percent of the *E. acervulina* ESTs share significant sequence identity with sequences in the *E. tenella* genome. Additionally, EST hits seem to be much different compared with those of *E. tenella*. One of the differences is the very low number of ESTs that encode putative microneme proteins. This study underlines the potential differences in the molecular aspects of 2 *Eimeria* species that in the past were thought to be highly similar in nature.

Coccidiosis in chickens is caused by 7 species of *Eimeria*. This complex disease is of major economic importance to poultry producers in the United States and worldwide (Allen and Fetterer, 2002; Williams, 2002). Currently, coccidiosis is treated predominantly with vaccines containing live (attenuated or virulent) oocysts derived from several *Eimeria* species (Vermeulen et al., 2001; Chapman et al., 2002). The use of chemotherapeutic agents represents the other predominant method of treating coccidiosis; however, drug resistance, cost of new drug development, and changes in laws regulating drug use (especially in Europe) have led to the increasing popularity of using vaccines to counter coccidiosis (Jenkins, 2001; Blake et al., 2006; Williams, 2006a). Because of the relatively high cost of producing live oocyst vaccines, and the need for alternative control measures, there has been some effort to produce a recombinant subunit vaccine. These efforts have not been successful (Jenkins, 2001). The difficulty in producing a subunit vaccine lies in identification of cross-reactive antigens that are also immunoprotective (Jenkins, 2001; Blake et al., 2006). The discovery of these antigens has been partially hampered due to the fact that the bulk of research concerning gene and antigen discovery using expressed sequence tag (EST) analysis, genomics, and proteomics has been limited to a single *Eimeria* species, i.e., *Eimeria tenella* (Li et al., 2003; Refeaga et al., 2003; Miska et al., 2004; de Venevelles et al., 2006). The wealth of information concerning the molecular characteristics of *E. tenella* is not surprising as recovery of this parasite is relatively easy because it infects the ceca. However, it has also been reported that *E. tenella* seems not to be as immunogenic as *E. maxima* or *E. acervulina* (Rose and Long, 1962), and it may not be as predominant as either of those species in the field (Jenkins et al., 2006).

To add a comparative component into the gene discovery data, we carried out a study investigating a relatively large number of ESTs generated from second-stage merozoites and some schizonts of *E. acervulina*. This species is highly abundant, and it has been found in every field sample analyzed in Europe, and in the United States (Jenkins et al., 2006; Peek and Landman, 2006; Williams, 2006b). Recent reports have also found that

drug resistance is common for *E. acervulina* (Peek and Landman, 2006; Williams, 2006b). The pathogenic effects of *E. acervulina* involve lesion formation in the duodenum and jejunum that result in weight loss and low feed conversion ratios (Long, 1973). Even though infections with *E. acervulina* typically do not result in mortality (Long, 1973), the parasite has a high reproductive capacity (Williams, 2001); therefore, high oocyst outputs are associated with *E. acervulina* infections. Because *E. acervulina* infects a different portion of the intestine than *E. tenella*, the recovery of the intracellular stages is difficult. This has led to a far larger body of molecular data being generated for *E. tenella*. For example, >34,000 ESTs from *E. tenella* have been deposited in the GenBank database, compared with 59 ESTs generated from *E. acervulina*. Analyzing ESTs from multiple species of *Eimeria* that are infectious to chickens is important because it will help identify genes and proteins whose sequence and expression are conserved. This analysis will help identify potential vaccine targets that are not only immunogenic but also cross-protective among *Eimeria* species.

MATERIALS AND METHODS

Host infection and parasite recovery

Chickens (Moyer's Hatcheries Inc., Quakertown, Pennsylvania) were infected with 15×10^5 *E. acervulina* oocysts, using gavage (Fetterer and Barfield, 2003). Infected birds were killed by cervical dislocation 89 hr postinfection (PI), and the duodenum and jejunum were removed. Isolation of *E. acervulina* merozoites was carried out as described previously (Xie et al., 1990; Miska et al., 2005). By microscopic examination, the preparation contained primarily merozoites with some schizonts. After isolation, the merozoites were pelleted and snap frozen at -70°C .

Recovery of RNA

Pelleted merozoites were resuspended in 10 ml of TRIzol reagent (Invitrogen, Carlsbad, California), and then they were vortexed for 1 min, followed by incubation on ice for 1 min (4 times). The remainder of the total RNA isolation procedure was carried out using the manufacturer's recommended protocol. RNA pellets were resuspended in DNase/RNase-free water (Invitrogen), and they were placed at -70°C until 5 mg of total RNA was obtained. Ethanol and 3.5 M NaAc were added to the RNA, and the samples were shipped on dry ice to the Amplicon Express facility (Pullman, Washington) for cDNA library construction.

cDNA library construction and screening

Briefly, mRNA was isolated from the total RNA, which was then reverse transcribed into cDNA using the ZAP-cDNA synthesis kit (Stratagene, La Jolla, California). The *E. acervulina* merozoite-derived

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cDNA was cloned into the Uni-ZAP XR Vector (Stratagene) incorporating the EcoRI and XhoI cloning sites at the 5' and 3' end of the insert, respectively. A mass in vivo excision was carried out producing a pBluescript SK (–) plasmid library (Stratagene). The effective library titer was approximately 8×10^6 colony-forming units/ml. The excised library was propagated in the SOLR *E. coli* strain, and stocks were frozen in Luria-Bertani (LB) medium containing 15% glycerol. The library was plated onto LB/Amp plates, and colonies were picked at random and grown overnight in LB/Amp medium. Plasmid DNA was purified using the QIAprep Spin Miniprep kit (QIAGEN, Valencia, California). By carrying out a restriction digest of 24 clones, it was estimated that the average insert size of this library was approximately 1.6 kilobases (kb). Clones were sequenced using universal M13 Forward and Reverse primers. All sequencing reactions were performed using the Big Dye sequencing kit version 3.1 (Applied Biosystems, Foster City, California) with nonisotopic dye terminators, and they were analyzed on an automated sequencer (3730xl DNA sequencer; Applied Biosystems).

Sequence analysis

All sequences generated were edited using the Sequencher 4.7 software (Gene Codes Corporation, Ann Arbor, Michigan). Vector sequences were located and trimmed from the 5' and 3' ends of each sequence. Additionally, any remaining vector sequences were identified by screening each sequence against the UniVec database (<http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html>). Reliability of the sequences was ascertained manually, and any low-quality sequence was removed. To identify clones overlapping in sequence, each was compared against all other sequences using the "assemble contigs" function of Sequencher 4.7. All unique sequences were compared with the nonredundant protein sequence database (nr), and Swiss-Prot database using BLASTX algorithm (Altschul et al., 1990). BLASTN algorithm (Altschul et al., 1990) was used to search the dbEST database (Boguski et al., 1993). Finally, sequences were compared against the *E. tenella* genome assembly available at the Sanger Institute (<http://www.sanger.ac.uk/cgi-bin/blast/submitblast/e.tenella/omni>) using BLASTN. A match between sequences was designated as significant if the *P* value was lower than 1×10^{-4} . All ESTs were submitted to the dbEST database under accession numbers EH385064–EH386434. All unique sequences were also deposited in a database located at http://129.24.144.92/et2/parsecity/zea_display.php, where results of each similarity search can be accessed directly.

RESULTS

Screening of cDNAs expressed by *E. acervulina* merozoites and schizonts

In total, 1,847 clones were sequenced from the *E. acervulina* cDNA library. From these, 1,426 high-quality sequences were obtained, representing 1,026 unique contiguous sequences (contigs); 205 contigs were composed of single pass sequences from multiple clones containing identical or partially overlapping sequences. A pdf file containing the overview of the similarity search analysis for each unique sequence generated in this study is available for download at <http://129.24.144.92/et2/TableIv2.pdf>. The length of the inserts found in the *E. acervulina* library was, on average, 1.6 kb. Therefore, in most cases, a single pass sequence, which on average resulted in 600 base pairs of high quality sequence, did not contain an entire open reading frame (ORF). However, based on the presence of a start codon and BLAST search results, we found that at least 45% of the clones contain entire ORFs. The redundancy in this sampling of clones was 28%. Each unique consensus sequence was compared with entries in the nr, and Swiss-Prot databases, using the blastx algorithm, which resulted in 40 and 32% of the sequences sharing significant identity (with a score of at least 1×10^{-4}) with previously described or hypothetical proteins from each of these databases, respectively. The BLASTN algorithm was used to

TABLE I. Breakdown by functional category of *E. acervulina* contigs that share significant sequence identity with entries in the nr and/or Swiss-Prot databases (n = 408 contigs).

Functional category	% of contigs
Hypothetical proteins of unknown function	22
Ribosomal proteins	15.7
Metabolism	15
DNA replication/transcription	10.5
Heat shock proteins/chaperonins	6.4
Transport	6.4
Translation	5.9
Structural	4.2
Protein degradation	3.9
Various enzymes	3.5
Cell cycle control	2
Signaling	1.5
Redox	1.5
Surface antigens	0.7
Microneme proteins	0.5

compare each sequence to all ESTs deposited in the dbEST database. The results of this search resulted in 29% of *E. acervulina* ESTs sharing significant sequences identity to other ESTs. Using a comparative approach, all unique *E. acervulina* sequences were also blasted against the *E. tenella* genome assembly using BLASTN; 47% of the unique *E. acervulina* sequences shared significant sequence identity with sequences in the *E. tenella* genome. The results of the database searches, including the name of the closest matching sequence, organism of origin, accession number, and *P* value are listed in a table that, because of its large size, is available for download at <http://129.24.144.92/gestac2/Table.I.pdf>.

Classification

To determine the type of cellular processes that *E. acervulina* merozoites may undergo, all contiguous sequences that shared significant sequence similarity with sequences in the nr and Swiss-Prot databases were converted into 15 functional categories that are shown in Table I. In total, 408 contigs were included in this analysis. Sequences matching hypothetical proteins of unknown functions that have been identified from other protists represent the most common classification. Sequences encoding ribosomal proteins represent the second most common classification. ESTs encoding proteins involved in metabolism, DNA replication, transcription, and transcript processing were also widely represented. Proteins that share homology with known chaperonins and other heat shock proteins represented 6.4% of the contigs in this study. Surprisingly, only 0.7 and 0.5% of *E. acervulina* contigs shared sequence homology with surface antigens and microneme proteins, respectively, found in other apicomplexans.

Most abundant ESTs

To determine which of the *E. acervulina* ESTs were most abundant, contigs containing the greatest number of overlapping sequences were examined (Table II). The most abundant contig (9) is made up of 16 ESTs and contains the full-length homolog of a protein designated SO7 described from *E. tenella*

TABLE II. Eighteen contigs containing the highest number of overlapping ESTs.

Contig no.	Putative ID	No. of ESTs	Length (nt)
9	Unknown protein RB-1a (SO7)	16	1,732
15	Unknown	11	1,024
31	Heat shock protein 70 (Hsp70)	9	1,758
40	Unknown	9	1,941
19	Elongation factor 1 α	8	806
77	α -Tubulin	8	785
6	Polyubiquitin	7	780
18	Ribosomal protein L7a	7	1,058
35	High mobility group protein	7	1,434
49	Purine nucleoside phosphatase	7	791
54	Gluteraldehyde-3-phosphate dehydrogenase	7	671
27	Unknown	6	1,836
29	Ribosomal protein S4	6	1,098
42	Surface antigen 3	6	1,356
75	Ribosomal protein L28	6	923
94	Elongation factor 1 α	6	823
112	Enolase	6	729
127	Ribosomal protein S16	6	825

(Danforth and Augustine, 1989; Liberator et al., 1989). Interestingly, another contig (9a) composed of 3 ESTs, spanning 1,045 nucleotides (nt), seems to also contain sequence that is very similar to that of contig 9; however, it is clear that these contigs were generated from 2 different transcripts. Four of the contigs shown in Table II encode proteins of unknown function, 3 of which did not share similarity to any sequences present in the 4 databases searched. Four of the highly abundant contigs encode ribosomal proteins; the other contigs had similarity to proteins associated with metabolism, translation, stress response, and structural elements of cells. These results are congruent with the overall classification of ESTs described above.

Results of querying dbEST

Of the *E. acervulina* ESTs described here, 287 shared significant identity with sequences in dbEST. The majority (64.5%) of these were most similar to ESTs generated from *E. tenella*. Three ESTs were identical in sequence to *E. acervulina* Open Reading Frame ESTs. An additional 10.5% of the ESTs were most similar in sequence to ESTs generated from other apicomplexans, whereas 16.7% were most similar to ESTs generated from "other" organisms, most commonly bacteria, plants, and vertebrates. Interestingly, 21 ESTs or 7.3% of *E. acervulina* identically matched ESTs from chickens. Two of these ESTs probably represent contamination of merozoite material with chicken tissue. However, 19 of these *E. acervulina* sequences were identical or highly similar to ESTs generated from a library made from intestinal intraepithelial lymphocytes, which were generated from chickens infected with *E. acervulina* and *E. maxima* (Min et al., 2005).

DISCUSSION

Because of the high proportion of unique genes present in apicomplexan parasites, screening of ESTs has proven to be a useful tool in gene discovery, expression profiling, comparative

genomics, and protein function prediction (Wan et al., 1999; Li et al., 2003; Klotz et al., 2005; Boyle et al., 2006). The 1,426 sequences from *E. acervulina* presented here represent the first EST analysis from this species. The cDNA library from which these sequences are derived seems to be of high quality, with inserts measuring an average of 1.6 kb, almost half of which encode full-length ORFs. Additionally, the library is not highly redundant, with almost 80% of the clones representing unique sequences. By comparing the 1,026 unique sequences to 4 databases, it seems that most of the sequences encode novel proteins of unknown function. This in itself is not unexpected because similar results have been observed in other apicomplexans (Boyle et al., 2006). One of the surprising findings of this study has been the low percentage of sequences that are significantly similar to *E. tenella* genome and ESTs sequences. Approximately 34,000 ESTs from various stages of the *E. tenella* life cycle have been deposited in dbEST (Wan et al., 1999; Ng et al., 2002; Li et al., 2003). Only 29% of the unique *E. acervulina* sequences shared significant similarity with entries in dbEST, and 64.5% of these were most similar to *E. tenella* ESTs. This translates to only 185 *E. acervulina* sequences from the total of 1,026 unique contigs (or 18% of total sequences) matching an *E. tenella* sequence. The comparison of *E. acervulina* ESTs to the *E. tenella* genome resulted in less than half of the ESTs finding a match in the genome. This represents the first comparative molecular analysis of these 2 species, and it suggests that they may be divergent. However, it is also likely that many more homologs will be identified when the *E. tenella* genome assembly is completed and annotated. Additionally, the presence of introns made this comparison more difficult, because intervening sequences will lower the E values of BLAST search results. Therefore, it is likely that the result of this comparison is skewed in a negative manner.

In *E. tenella* merozoites, some of the most abundantly expressed transcripts (10–20% of all ESTs) encode microneme proteins, which play an important role in parasite invasion (Tomley and Soldati, 2001; Ng et al., 2002; Li et al., 2003), and they are limited to apicomplexans. In contrast, only 2 *E. acervulina* ESTs were found (which represents 0.002% of the total unique sequences reported) that encode homologs of *E. tenella* microneme 5 (Brown et al., 2000), and micronemal protein 4 from *Toxoplasma gondii* (Brecht et al., 2001). This is interesting because these 2 proteins are the only Apple domain-containing microneme proteins that have been so far identified in these 2 taxa (Tomley and Soldati, 2001). It will be very interesting to further investigate the microneme proteins in *E. acervulina* to determine whether the repertoire of these proteins is limited, and whether the expression of these genes is significantly lower than observed in *E. tenella*, or whether these results were produced due to a bias in clone composition of the cDNA library.

Although ESTs encoding microneme proteins seem to be underrepresented in *E. acervulina* merozoites, the most abundant sequence found in this study encodes a homolog of SO7, an antigen that has been investigated as a possible cross-species protective vaccine candidate (Danforth and Augustine, 1989). This protein seems to also be associated with refractile bodies (RBs), which are organelles specific to the Eimeriidae whose function is unknown (de Venevelles et al., 2006). Interestingly, RBs are not likely to be present in second-stage merozoites

(Hammond et al., 1970). However, the library from which these clones were isolated was collected mainly from second-generation merozoites; therefore, it will be interesting to investigate whether this protein has several functions and whether its expression profile is different in *E. acervulina* and *E. tenella*. Additionally, 2 unique sequences were identified that encode the *E. acervulina* SO7 homolog. These most likely represent a splice variant; however, it should also be investigated whether *E. acervulina* contains multiple copies of SO7 in its genome.

Altogether, we found that screening *E. acervulina* merozoite library yielded many novel genes for which homologous sequences have not yet been described in other apicomplexans. Also, EST encoding genes that are abundantly expressed in *E. tenella* merozoites, such as those encoding microneme proteins, are underrepresented in the *E. acervulina* library. The results of the present study indicate that potential molecular differences exist between these 2 seemingly similar species. Even though *E. tenella* is the most studied member of the species that cause poultry coccidiosis, it is imperative that multiple *Eimeria* species be investigated both for basic comparative analysis and for development of effective coccidiosis control. It is highly likely that at the molecular level, poultry *Eimeria* species are quite diverse and should not be treated as a single organism.

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